DESCRIPTION

METHOD AND KIT FOR DETECTING PROLIFERATIVE DISEASES CAUSING SCLEROSIS, PROPHYLACTIC AND/OR THERAPEUTIC AGENT FOR PROLIFERATIVE DISEASES CAUSING SCLEROSIS, AND METHOD AND KIT FOR IDENTIFYING SUBSTANCES EFFECTIVE IN PREVENTING AND/OR TREATING PROLIFERATIVE DISEASES CAUSING SCLEROSIS

TECHNICAL FIELD

The present invention relates to a method of detecting proliferative diseases causing sclerosis and a kit therefor; a prophylactic and/or therapeutic agent for proliferative diseases causing sclerosis, as well as a method of identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis and a kit therefor.

BACKGROUND ART

α1 type IV collagen (Col4) is a major component of the vascular basement membrane that lies beneath the endothelium and surrounds medial smooth muscle cells, and the overproduction of Col4 plays a crucial role in the process of diabetic angiopathy, arteriosclerosis and aging-related diseases. Prolonged exposure to hyperglycemia is now recognized as a significant causal factor of diabetic complications (non-patent documents 1 and 2). Excessive advanced glycation end-products (AGEs) produced as a result of hyperglycemia are known to induce a variety of cellular events in vascular cells and other cells, possibly through several functional AGEs receptors, thereby modulating the disease processes (non-patent documents 3, 4 and 5). AGEs have been recently accepted as playing an important role, not only in diabetic complications, but also in arteriosclerosis caused by aging (non-patent documents 6 and 7). Moreover, a truncated, soluble form of the receptor for AGEs was reported to inhibit the progress of accelerated diabetic atherosclerosis (non-patent document 8).

Morphologically, the progress of diabetic nephropathy is characterized by progressive thickening of the glomerular basement membrane (GBM) and by expansion of the mesangial extracellular matrix (ECM). Since Col4 is a major component of the thickened GBM and expanded ECM, it is important to clarify how Col4 is regulated at the transcriptional level in the diabetic state. The 130-bp bidirectional promoter of Col4 contains a large stem-loop structure (CIV) which has been shown to interact with several DNA binding proteins (non-patent documents 9). Using a gel mobility shift assay, the

present inventors previously reported that an unknown protein binds to the CIV site only when Col4 is induced by the exposure to AGEs (non-patent document 10).

Both mesangial cell proliferation and glomerulosclerosis are major pathological features in progressive glomerular disorders. The fact that mesangial cell proliferation is observed in many glomerular sclerosing diseases suggests that this process is important in progressive glomerular disorders (non-patent document 11 (A1), non-patent document 12 (A2)). Both events are concomitantly observed in most of glomerular diseases, but it is not clear how cell proliferation is involved in the progress of glomerulosclerosis.

Platelet derived growth factor (PDGF) was shown as a critical mitogen for mesangial cells *in vitro* and *in vivo* (non-patent document 13 (3A), non-patent document 14 (4A)). Not only in experimental models but also in human glomerular diseases, it has been proved that PDGF plays a key role in the progress of glomerulosclerosis (non-patent document 13 (A3)). PDGF-BB was also reported to be essential for mesangial cell proliferation (non-patent document 15(A5)), which is followed by development of glomerulosclerosis in a remnant kidney model (non-patent document 16 (A6)). Introduction of neutralizing anti-PDGF antibody markedly ameliorated both mesangial proliferation and glomerulosclerosis in a rat glomerulonephritis model (non-patent document 17 (A7)), but little was known about the mechanism how inhibition of cell proliferation reduces glomerular sclerotic lesions.

Non-patent document 1:

The Diabetes Control and Complications Trial Research Group. *N. Engl. J. Med.* 329, 977-986 (1993).

Non-patent document 2:

UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 352, 837-853 (1998)

Non-patent document 3:

H. Vlassara, *et al.*, *Proc. Natl. Acad. Sci. USA* 91, 11704-11708 (1994).

Non-patent document 4: M. Brownlee, A. Cerami, H. Vlassara, *N. Engl. J. Me*d. 318, 1315-1321 (1988).

Non-patent document 5:

T. Doi, *et al.*, *Proc. Natl. Acad. Sci. U S A* 89, 2873-2877 (1992).

Non-patent document 6:

H. Vlassara, *et al.*, *Proc. Natl. Acad. Sci. U S A* 89, 12043-12047 (1992).

Non-patent document 7:

M. S. Huijberts, *et al.*, *J. Clin. Invest.* 92, 1407-1411 (1993).

Non-patent document 8:

S. L. Park, *et al.*, *Nature Med.* 9, 1025-1031 (1998)
Non-patent document 9:
L. A. Bruggeman, P. D. Burbelo, Y. Yamada, P. E. Klotman, *Oncogene* 7, 1497-1502 (1992).
Non-patent document 10:
N. Iehara, H. Takeoka, Y. Yamada, T. Kita, T. Doi, *Kidney Int.* 50, 1166-1172 (1996).
Non-patent document 11:
Fogo A, Ichikawa I. Evidence for the central role of glomerular growth promoters in the development of sclerosis. Semin Nephrol. 1989 Dec; 9(4):329-42.
Non-patent document 12:
Striker LJ, Doi T, Elliot S, Striker GE. The contribution of glomerular mesangial cells to progressive glomerulosclerosis. Semin Nephrol. 1989 Dec; 9(4):318-28. Review.
Non-patent document 13:
Floege J, Johnson RJ: Multiple roles for platelet-derived growth factor in renal disease. Miner Electrolyte Metab 21: 271-282, 1995
Non-patent document 14:
Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ: Receptor-specific increase in extracellular matrix production by mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. Proc Natl Acad Sci USA 89: 2873-2877, 1992
Non-patent document 15:
Barnes JL, Hevey KA. Glomerular mesangial cell migration in response to platelet-derived growth factor. Lab Invest. 1990 Mar; 62(3):379-82.
Non-patent document 16:
Floege, J., Burns, M. W., Alpers, C. E., Yoshimura, A., Pritzl, P., Gordon, K., Seifert, R. A., Bowen-Pope, D. F., Couser, W. G., and Johnson, R. J.: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. Kidney Int. 41: 297-309, 1992
Non-patent document 17:
Johnson, R. J., Raines, E. W., Floege, J, et al: Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. J Exp Med 175: 1413-1416, 1992

DISCLOSURE OF THE INVENTION

PROBLEM FOR SOLUTION BY THE INVENTION

Diabetic nephropathy is the leading cause of end-stage renal failure. Type IV

collagen is a principal component of the vascular basement membrane and the mesangial matrix of renal glomeruli, and plays a crucial role in the process of diabetic anigiopathy. However, what is directly involved in the overproduction of type IV collagen in diabetic state is unknown. It is an object of the present invention to identify the substance that is directly involved in the overproduction of type IV collagen and to demonstrate that the substance plays a critical role as a causative of diabetic nephropathy. It is another object of the present invention to provide a method and a kit for detecting diabetic nephropathy using the substance that is directly involved in the overproduction of type IV collagen. It is still another object of the invention to provide uses of those substances having an inhibitory effect on the expression of the substance that is directly involved in the overproduction of type IV collagen. It is still another object of the invention to provide a method and a kit for identifying substances effective in preventing and/or treating diabetic nephropathy; a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix; and a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen.

Further, the present invention aims at demonstrating the effect of administration of anti-PDGF β receptor antibody (APB5) (which inhibits activation by PDGF-B chain) on rat glomerulonephritis to thereby demonstrate *in vivo* and *in vitro* that the PDGF signal transduction pathway is regulating both glomerular cell proliferation and glomerulosclerosis. The present invention also aims at providing a method and a kit for detecting proliferative diseases causing sclerosis, using those substances involved in glomerular cell proliferation and glomerulosclerosis. Further, the present invention aims at providing uses of substances which have an inhibitory effect on the expression of those substances involved in glomerular cell proliferation and glomerulosclerosis. Still further, the present invention aims at providing a method and a kit for identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis; a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix; and a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen.

MEANS TO SOLVE THE PROBLEM

The present inventors have identified Smad1 as a substance that is directly involved in the overproduction of type IV collagen and demonstrated that Smad1 plays a critical role as a causative of diabetic nephropathy. The present inventors have also examined the expression of Smad1 and activin receptor-like kinase 1 (ALK1) in renal glomeruli of healthy persons and diabetic nephropathy patients, and found that while the expression of Smad1 and

ALK1 in diabetic nephropathy patients is proportional to the severity of sclerosis lesions, the expression of Smad1 and ALK1 is hardly observed in healthy persons. Further, the present inventors have also found that the expression of BMP2 and BMP4 (which regulate the expression of Smad1) increases in the presence of AGEs stimulation.

On the other hand, the fact that mesangial cell proliferation is observed in many glomerul sclerosing diseases suggests that this process is important in progressive glomerular disorders. However, relations between the cell proliferation and glomerulosclerosis are not clear. Recently, the present inventors showed that the overexpression of type IV collagen (Col4), one of major components of glomerulosclerosis, is transcriptionally regulated by Smad1 in diabetic glomerulosclerosis. In this study, the present inventors have demonstrated the effect of administration of anti-PDGF β-receptor antibody (APB5) (which inhibits activation by PDGF-B chain) on rat glomerulonephritis and thereby demonstrated *in vivo* and *in vitro* that the PDGF signal transduction pathway is regulating both glomerular cell proliferation and glomerulosclerosis.

An experimental model of mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody. In Thy1 GN, mesangial cell proliferation and expression of Col4 peaked at day 6. Immunohistochemical staining was performed to examine the expression of Smad1, phosphorylated Smad1 (pSmad1) and phosphorylated STAT3 (pSTAT3). The peak of glomerular Smad1 expression occurred at day 6, which was consistent with the peak of mesangial proliferation. Glomerular pSmad1 expression was upregulated from day 1 of Thy1 GN, and the peak of glomerular pSmad1 expression occurred at day 4 of the disease. In APB5-treated groups, both mesangial proliferation and glomerulosclerosis were reduced significantly. Smad1, pSmad1 and pSTAT3 expressions were also significantly reduced by administration of APB5 at every point examined. APB5 treatment reduced mesangial cell proliferation in association with reduction in pSmad1, pSTAT3 and Col IV protein expressions *in vitro*. Introduction of dominant negative STAT3 decreased the expression of Col4 significantly in cultured mesangial cells. These data suggest that activation of STAT3 and Smad1 is involved in the progress from mesangial cell proliferation to glomerulosclerosis.

The present invention has been achieved based on these findings.

The subject matters of the present invention are as described below.

(1) A method of detecting proliferative diseases causing sclerosis, comprising measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1,

activin receptor-like kinase 3 and bone morphogenetic proteins in a biological sample.

(2) A method of evaluating the degree of progress and/or the efficacy of treatment of proliferative diseases causing sclerosis, comprising measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins in a biological sample.

(3) A kit for detecting proliferative diseases causing sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins in a biological sample.

(4) A kit for evaluating the degree of progress and/or the efficacy of treatment of proliferative diseases causing sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins in a biological sample.

(5) A method of detecting diabetic nephropathy, comprising measuring the expression of Smad1 and/or a substance having Smad1-activating effect in a biological sample.

(6) A method of evaluating the degree of progress and/or the efficacy of treatment of diabetic nephropathy, comprising measuring the expression of Smad1 and/or a substance having Smad1-activating effect in a biological sample.

(7) A kit for detecting diabetic nephropathy, comprising a reagent(s) for measuring the expression of Smad1 and/or a substance having Smad1-activating effect.

(8) A kit for evaluating the degree of progress and/or the efficacy of treatment of diabetic nephropathy, comprising a reagent(s) for measuring the expression of Smad1 and/or a substance having Smad1-activating effect.

(9) A prophylactic and/or therapeutic agent for proliferative diseases causing sclerosis, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(10) A drug inhibiting the increase of extracellular matrix, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(11) A drug inhibiting the expression of α1 type IV collagen, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance

selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(12) A method of identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(13) A method of identifying substances effective in inhibiting the increase of extracellular matrix, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(14) A method of identifying substances effective in inhibiting the expression of α1 type IV collagen, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(15) A kit for identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(16) A kit for identifying substances effective in inhibiting the increase of extracellular matrix, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

(17) A kit for identifying substances effective in inhibiting the expression of α1 type IV collagen, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

EFFECT OF THE INVENTION

According to the present invention, Smad1 was identified as a substance directly involved in the overproduction of type IV collagen, and it was demonstrated that Smad1 has a critical role as a causative of diabetic nephropathy. With these findings, it has become possible to detect diabetic nephropathy; besides, a prophylactic and/or therapeutic for diabetic nephropathy, a drug inhibiting the increase of extracellular matrix, and a drug inhibiting the expression of α1 type IV collagen have been provided. Further, according to the present invention, there have been provided a method and a kit for identifying substances

effective in preventing and/or treating diabetic nephropathy; a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix; and a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen.

Further, according to the present invention, it has been demonstrated that the activation of STAT3 and Smad1 is in a key pathway regulating the interaction between cell proliferation and glomerulosclerosis which are the two phenomena observed in progressive glomerular disorders. With this finding, it has become possible to detect proliferative diseases causing sclerosis; besides, a prophylactic and/or therapeutic for proliferative diseases causing sclerosis, a drug inhibiting the increase of extracellular matrix, and a drug inhibiting the expression of α1 type IV collagen have been provided. Further, according to the present invention, there have been provided a method and a kit for identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis; a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix; and a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen.

The present specification encompasses the contents described in the specification and/or the drawings of Japanese Patent Application No. 2003-319538 based on which the present patent application claims priority.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows activation of Col4 promoter by Smad1. (A): Chromatin immunoprecipitation was carried out using cultured mesangial cells in the presence of AGEs or BSA (control), using the indicated antibodies. PCR was performed using primers for CIV-1 motif. The results of one experiment out of three independent experiments are shown. (B): Cells were cotransfected with a vector containing CIV-1-lacZ reporter plasmid together with either a wild type Smad1 vector or a mock vector (Mock), and CMV-LUC as an internal control. Cell extracts were analyzed by Western blotting using anti-Smad1 and anti-pSmad1 antibodies. The results of one experiment out of three independent experiments are shown. (C): After 48 hours, cultured cells were lysed, followed by measurement of β-galactosidase and luciferase activities. Values are the averages of triplicate determinations with SD.

Fig. 2 shows Smad1 expression changing dynamically on exposure to AGEs. (A): RNase protection assay was performed to examine the time course of Smad1 and Col4 mRNA expressions in mesangial cells treated with AGEs or BSA. Continuous exposure to

AGEs promotes Smad1 expression continuously in parallel with increase in Col4 expression. The results of one experiment out of three independent experiments are shown. (B): Immunofluorescence photographs of mesangial cells cultured for 72 hr or 120 hr in the presence of AGEs or BSA. Data from one of three independent experiments are shown. (C): Smad1 and pSmad1 were analyzed by Western blotting in cells cultured for 72 hr in the presence of AGEs or BSA. Data from one of three independent experiments are shown.

Fig. 3 shows the effect of an antisense oligo specific to Smad1 in mesangial cells. (A): After 72 hr-incubation with AGEs, mesangial cells were incubated for 16 hr in a medium containing an antisense oligo to Smad1 or 4-mismatch oligo (control). The antisense oligo-treated mesangial cells were immunofluorescently stained with anti-Smad1 antibody (green), and further stained with DAPI (blue). Data from one of three representative experiments are shown. (B): The antisense oligo to Smad1 or 4-mismatch oligo (control) was introduced into mesangial cells treated with AGEs. Data from one of three independent experiments are shown. (C): The antisense oligo to Smad1 inhibits the upregulation of Smad1 expression and, at the same time, the upregulation of Col4 expression. Data from one of three independent experiments are shown.

Fig. 4 shows the detection of Smad1 and ALK1 expressions in human patients with diabetic nephropathy. Glomeruli from 5 diabetic patients and 3 non-diabetic patients were immunohistochemically stained with anti-Smad1 and anti-ALK1 antibodies. Smad1 and ALK1 expressions were markedly detectable in the glomeruli of diabetic patients, but not detected in non-diabetic patients. All sections were counterstained with hematoxylin. Magnification is x400 for all photographs.

Fig. 5 shows the results of comparison between mRNA expression levels in mesangial cells cultured in the presence of AGEs and corresponding mRNA expression levels in mesangial cells cultured in the presence of BSA.

Fig. 6 shows the results of determination by Western blotting of urinary BMP2 levels in diabetic nephropathy patients.

Fig. 7 shows the results of determination by Western blotting of the expression of BMP2 and BMP4 in the presence of chronic stimulation with TGF-β signal.

Fig. 8 is a schematic drawing of the signal transduction pathway based on the results of Example 1.

Fig. 9 is microscopic images showing diffuse increase of the mesangial matrix and expansion of the mesangial area in Thy1 GN rats. Overexpression of Col4 was observed in the expanded mesangial area by immunohistochemical staining with anti-Col4 antibody. APB5 reduced both mesangial proliferation and Col4 expression. Thy1 GN glomeruli were

significantly positive in PDGF-B chain and PDGF β receptor. APB5 also reduced these overexpressions. A-C: PAM; D-F: Col4; G-H: PDGF-B chain; J-K: PDGF β receptor; A, D, G and J: normal control rats; B, E, H and J: disease control rats at day 6; C, F, I and L: APB5-treated rats at day 6.

Fig. 10. Quantitation of histological changes and effects of APB5 administration in Thy1 GN. A: Glomerular cell number. Increase in glomerular cell number is observed in Thy1 GN groups. B: PCNA positive cell number in Thy1 GN. PCNA-positive cell number in the glomeruli of APB5-treated rats was significantly reduced at each point examined. C: Mesangial matrix expansion. Mesangial matrix increase was observed at Day 6 in Thy1 GN rats. APB5 significantly reduced mesangial matrix increase at each point examined. D: Expression of type IV collagen. In the control group, Col4 was strongly positive in the expanded mesangial area. APB5 significantly reduced Col4 expression. *P<0.001 vs. control group; **P<0.001 vs. APB5 non-treated disease control group.

Fig. 11. Immunohistochemical staining of Smad1, phosphorylated Smad1 and phosphorylated STAT3 in Thy1 GN. Smad1, phosphorylated Smad1 and phosphorylated STAT3 expressions showed a surprising increase in immunohistochemical staining of the glomeruli of Thy1 GN rats. Phosphorylated Smad1 was observed remarkably at the same site as nuclei were observed in Thy1 GN rats. APB5 treatment brought significant reduction in each of these substances. A-C: Smad1; D-F: phosphorylated Smad1; G-I: phosphorylated STAT3; A, D and G: normal control rats; B, E and H: untreated Thy1 rats at day 6; C, F and I: APB5-treated rats at day 6.

Fig. 12. Time course of Smad1, pSmad1 and pSTAT3 expressions. Day 0, day 1, day 2, day 4, day 6 and day 12 renal sections from Thy1 GN rats were immunohistologically stained with anti-Smad1, anti-pSmad1 and anti-pSTAT3 antibodies. A: Smad1 expression in Thy1 GN. Smad1 expression peaked at Day 6 and was calmed down at day 12. B: Time course of the ratio of pSmad1 positive cells to the total glomerular cell number. pSmad1 expression peaked at Day 4. C: Time course of pSTAT3 expression. The ratio of pSTAT3 positive portion to the mesangial area increased up to Day 6, and was calmed down at day 12. *P<0.001 vs. control group; **P<0.001 vs. each examination point.

Fig. 13. Effects of APB5 treatment on Smad1, pSmad1 and pSTAT3 expressions. The results of immunohistological staining and quantitation of Smad1, pSmad1 and pSTAT3 expressions revealed that these proteins were reduced by APB5 treatment as Col4 expression in mesangial matrix and glomeruli was reduced. A: Smad1 expression. B: pSmad1 expression. C: pSTAT3 expression. *P<0.01 vs. APB5 non-treated disease control.

Fig. 14. Effects of APB5 *in vivo*. A: Inhibitory effect of APB5 on mesangial cell proliferation. Addition of PDGF-B increased the proliferation of mesangial cell, and APB5 significantly inhibited this proliferation. $*P<0.05$ vs. control; $**P<0.05$ vs. PDGF-B stimulated control. B: Western blot analysis revealed that pSTAT3, pSmad1 and Col4 protein expressions were reduced by addition of APB5. The results of one experiment out of three independent experiments are shown.

Fig. 15. Western blot analysis of gene-transfected mesangial cells. pSmad1 and Col4 protein expressions were reduced by dominant negative STAT3. The results of one experiment out of three independent experiments are shown.

Fig. 16 is a schematic drawing of the signal transduction pathway based on the results of Examples 1 and 2.

Fig. 17 shows the results of Western blotting on urine samples from patients and healthy persons using anti-ALK-1 antibody as a primary antibody. Lanes 1-5: diabetic nephropathy patients; lane 6: patient with mitochondrial disease in which diabetes is complicated with a sclerosing, renal proliferative disease; lanes 7 and 8: patients with diabetes complicated with a non-sclerosing renal disease; lanes 9 and 10: healthy persons.

Fig. 18 shows the results of Western blotting on urine samples from a diabetic nephropathy patient under treatment, using anti-ALK-1 antibody as the primary antibody. Electrophoregrams taken at one week intervals are shown starting from the utmost left lane.

Fig. 19 shows the results of Western blotting on urine samples from patients and healthy persons using anti-Smad1 antibody as a primary antibody. Lanes 1-5: diabetic nephropathy patients; lane 6: patient with mitochondrial disease in which diabetes is complicated with a sclerosing, renal proliferative disease; lanes 7 and 8: patients with diabetes complicated with a non-sclerosing renal disease; lanes 9 and 10: healthy persons.

BEST MODE FOR CARRYING OUT THE INVENTION

1. Method and Kit for Detecting Diabetic Nephropathy

The present invention provides a method of detecting diabetic nephropathy, comprising measuring the expression of Smad1 and/or a substance having Smad1-activating effect in a biological sample.

The biological sample may be any biological sample as long as Smad1 and/or a substance having Smad1-activating effect is detectable therein. Specific examples of the biological sample which may be used in the invention include renal tissue sections, blood, sera and urine.

Nine Smad proteins (Smad1 to Smad9) have been identified in mammals, and

Smad1 is known as a member of the bone morphogenetic protein (BMP) signal transduction pathway. BMPs regulate the transcription of target genes through activin receptor kinase 2, 3 and 6 (AKL2, ALK3 and ALK6) (Zwijsen A. et al., FEBS Letters 546, 2003, 133-139). In addition to Smad1, Smad5 and Smad8 are also involved in the BMP signaling specifically. Further, Smad2 and Smad3 are said to be involved in the TGF-β/activin signaling specifically. On the other hand, it has been elucidated that Smad1 transduces TGF-β signals through activin receptor-like kinase 1 (ALK1) to thereby regulate the transcription of target genes in endothelial cells and hematopoietic cells (Goumans MJ. et al., EMBO J., 2002, Apr 2, 21(7), 1743-53). This means that two major signal transduction pathways (BMP pathway and TGF-β pathway) exist in which transcription of target genes is regulated by activation of Smad1 (Fig. 8). However, sufficient examination has not been made yet as to a pathway of which combination is the most important.

The "substance having Smad1-activating effect" may be any substance as long as it is capable activating Smad1. For example, substances such as activin receptor-like kinase 1 (ALK1) and activin receptor-like kinase 3 (ALK3) which activate Smad1 directly may be given. Alternatively, substances such as bone morphogenetic proteins (BMPs) which activate Smad1 indirectly through activation of activin receptor kinases (ALKs) may be given.

It is clear from the study of the present inventors (Example 2) that PDGF also activates Smad1 though directly or indirectly is not known.

The expression "activates Smad1" means to phosphorylate serine residues of Smad1 and/or to translocate Smad1 into the nucleus.

Activin receptor-like kinase 1 (ALK1) is one of the type I receptors which bind to TGF-β family proteins and is known to activate Smad1 (Chen YG, et al., Smad1 recognition and activation by the ALK1 group of transforming growth factor-β family receptors J.Biol.Chem. Vol.274, No.6, 3672-3677, 1999). ALK1 is expressed highly in the placenta, lung and vascular endothelial cells in human, and mutations of ALK1 result in human hereditary hemorrhagic telangiectasia (HHT) type II, also known as Osler-Rendu-Weber syndrome (non-patent document 17).

Activin receptor-like kinase 3 (ALK3), also known as BMPR-IA, is one of the type I receptors which bind to BMP family proteins, and is a serine-threonine receptor. ALK3 bound to BMPs activates Smad1, Smad5 and Smad8 and carries out the transduction of signals into the nucleus.

Bone morphogenetic proteins (BMPs) are a member of TGF-β superfamily and involved in bone morphogenesis as well as development of four limbs and differentiation of

the nerve system in the developmental stage. However, several reports that BMPs are involved in the regulation of development of the metanephros have been made recently and attracted attention. The kidney develops from the intermediate mesoderm and is formed through the three stages of pronephros, mesonephros and metanephros. Most of the pronephros and mesonephros undergo retroplasia eventually; the kidney which functions in mammalian adults is the metanephros. Transcripts for BMPs and their receptors have been localized in the developing metanephros. BMP2, BMP4 and BMP7 have direct or indirect roles in regulation of ureteric branching morphogenesis and branch formation *in vitro*. *In vivo*, it is reported that renal phenotypes vary between BMP7 null mutation-homozygous mutant mice and BMP4 null mutation-heterozygous mutant mice (Martinez G, et al, Int J Dev Biol. 2002; 46(4):525-33).

TGF-β has diversified effects and plays important roles in proliferation/differentiation of various cells, production of extracellular matrix, apoptosis, immune system, and so forth. TGF-β binds to receptors on cell surfaces to thereby transduce its signals into cells. A series of Smad protein molecules play important roles in the intracellular signal transduction.

To date, a pathway in which TGF-β activates Smad2 and Smad3 through ALK5 under hyperglycemic conditions to thereby bring about the overproduction of extracellular matrix such as α1 type IV collagen has been considered to be involved in the development and progress of diabetic nephropathy (Jin H. et al., Kidney International, 63, 2003, 2010-2019). However, the present study shows for the first time that there exists a pathway which brings about overproduction of extracellular matrix through Smad1 under hyperglycemic conditions.

The expression of Smad1 and/or a substance having Smad1-activating effect may be measured at the nucleic acid level (i.e. mRNA expression) and/or the protein level.

With respect to the measurement at the nucleic acid level, total RNA may be extracted from a biological sample, and then the mRNA of Smad1 and/or a substance having Smad1-activating effect may be measured by RT-PCR using a pair of appropriate primers. These primers may be designed so that a specific region in sequences such as the nucleotide sequence for human-derived Smad1 mRNA available as NM_005900 in NCBI Refseq database (SEQ ID NO: 1); the nucleotide sequence for human-derived activin receptor-like kinase 1 mRNA available as NM_000020 in NCBI Refseq database (SEQ ID NO: 2); the nucleotide sequence for BMP2 mRNA available as ACCESSION NM_001200 VERSION NM_001200.1 in GenBank database (SEQ ID NO: 3); and the nucleotide sequence for BMP4 mRNA available as ACCESSION NM_001202 VERSION NM_001202.2 in

GenBank database (SEQ ID NO: 4) is amplified specifically. Examples of nucleotide sequences for appropriate primer pairs are as described below.

RT-PCR to amplify Smad1 mRNA specifically:

Forward primer: 5'-ACTACCACCACGGCTTTCAC-3' (SEQ ID NO: 5)

Reverse primer: 5'-AATAGGATTGTGGGGTGAGC-3' (SEQ ID NO: 6)

RT-PCR to amplify ALK1 mRNA specifically:

Forward primer: 5'-ccgtcaagatct tctcctcg-3' (SEQ ID NO: 7)

Reveres primer: 5'-tcatgtctgaggcgatgaag-3' (SEQ ID NO: 8)

RT-PCR to amplify BMP2 mRNA specifically:

Forward primer: 5'- cccagcgtgaaaagagagac-3' (SEQ ID NO: 9)

Reverse primer: 5'- gagaccgcagtccgtctaag-3' (SEQ ID NO: 10)

RT-PCR to amplify BMP4 mRNA specifically:

Forward primer: 5'- tgagcctttccagcaagttt -3' (SEQ ID NO: 11)

Reverse primer: 5'- cttccccgtctcaggtatca -3' (SEQ ID NO: 12)

Alternatively, total RNA may be extracted from a biological sample, and then the mRNA of Smad1 and/or a substance having Smad1-activating effect may be measured by Northern hybridization using an appropriate probe. The appropriate probe may be designed based on sequences such as the nucleotide sequence for human-derived Smad1 mRNA available as NM_005900 in NCBI Refseq database (SEQ ID NO: 1); the nucleotide sequence for human-derived activin receptor-like kinase 1 mRNA available as NM_000020 in NCBI Refseq database (SEQ ID NO: 2); the nucleotide sequence for BMP2 mRNA available as ACCESSION NM_001200 VERSION NM_001200.1 in GenBank database (SEQ ID NO: 3); and the nucleotide sequence for BMP4 mRNA available as ACCESSION NM_001202 VERSION NM_001202.2 in GenBank database (SEQ ID NO: 4) so that it specifically hybridizes to a part or the entire region of such sequences. The probe may be labeled with a substance such as ^{32}P.

With respect to the measurement at the protein level, Smad1 and/or a substance having Smad1-activating effect may be measured by a method such as Western blotting, ELISA or immunohistochemical analysis using, for example, anti-Smad1 antibody and/or antibody to the substance having Smad1-activating effect. The anti-Smad1 antibody and/or antibody to the substance having Smad1-activating effect may be labeled with a fluorescent dye, enzyme, heavy metal, or the like (direct method). Alternatively, instead of labeling these antibodies, antibodies (secondary antibodies) specific to these antibodies (primary antibodies) may be labeled with a fluorescent dye, enzyme, heavy metal, or the like (indirect method). Preferably, these antibodies are immobilized on solid carriers such as test sections

or latex particles.

The expression "measuring the expression of Smad1 and/or a substance having Smad1-activating effect" encompasses to detect the presence or absence of the expression of Smad1 and/or a substance having Smad1-activating effect and to quantitate the expression level of Smad1 and/or a substance having Smad1-activating effect.

According to the present invention, it is possible to detect diabetic nephropathy. Briefly, the expression of Smad1 and/or a substance having Smad1-activating effect indicates the onset of diabetic nephropathy. Conventionally, measurement of urinary type IV collagen and urinary albumin has been used in the diagnosis of diabetic nephropathy. The present invention may supersede or supplement such measurement.

Further, according to the present invention, it is possible to evaluate the degree of progress and/or the efficacy of treatment of diabetic nephropathy. Briefly, the expression level of Smad1 and/or a substance having Smad1-activating effect is proportional to the severity of diabetic nephropathy. When the treatment of diabetic nephropathy is effective, the expression level of Smad1 and/or a substance having Smad1-activating effect decreases keeping pace with the recovery of the patient.

Diabetic nephropathy is one of the microangiopathic disorders caused by chronic hyperglycemic conditions. Pathologically, diabetic nephropathy presents thickening of the renal glomerular basement membrane, expansion of the mesangial area and glomerulosclerosis lesions; clinically, diabetic nephropathy presents symptoms such as proteinuria (microalbuminuria), hypertension or edema. Ultimately, diabetic nephropathy patients often develop renal failure. In diabetes, abnormalities such as arteriolosclerosis, denaturing/fibrosing of the tubulointerstitium, etc. are recognized in tissues other than the glomeruli, and these abnormalities make glomerular lesions even worse. Therefore, it is possible to define the pathology in which proteinuria, hypertension and renal function disorders are gradually progressing after a specific period of diabetes, as nephropathy.

Recently, more than 30% of the primary diseases of those patients who newly receive dialysis treatment because of their end-stage renal failure is diabetic nephropathy, and this ratio is still increasing. Further, prognosis of these patients after the introduction of dialysis is not necessarily good, which is a big problem in medical treatment. Therefore, it has become an important problem to elucidate the mechanism of development and progress of diabetic nephropathy and to develop diagnosis and treatment thereof (Japanese Journal of Clinical Medicine vol. 55, 1997 special issue "Diabetes" (1)).

The present invention also provides a kit for detecting diabetic nephropathy, comprising a reagent(s) for measuring the expression of Smad1 and/or a substance having

Smad1-activating effect.

Further, the present invention provides a kit for evaluating the degree of progress and/or the efficacy of treatment of diabetic nephropathy, comprising a reagent(s) for measuring the expression of Smad1 and/or a substance having Smad1-activating effect.

Examples of reagents for measuring the expression of Smad1 and/or a substance having Smad1-activating effect include, but are not limited to, a pair of primers capable of amplifying a specific region of the nucleotide sequence of Smad1 mRNA; a pair of primers capable of amplifying a specific region of the nucleotide sequence of the mRNA of a substance having Smad1-activating effect; a probe capable of hybridizing to a part or the entire region of Smad1 mRNA; a probe capable of hybridizing to a part or the entire region of the mRNA of a substance having Smad1-activating effect; an antibody to Smad1; and an antibody to a substance having Smad1-activating effect. These primer pairs and antibodies are as described above.

The kit of the invention may further comprise reverse transcriptase, DNA polymerase, RNase-free water, buffers, control mRNA, control primer pair, dNTP mix, instructions, and so forth (when the kit is intended to measure the expression of Smad1 and/or a substance having Smad1-activating effect at the nuclear acid level using a primer pair).

Alternatively, the kit of the invention may further comprise a transcription buffer, blocking reagent, washing solutions, instructions and so forth (when the kit is intended to measure the expression of Smad1 and/or a substance having Smad1-activating effect by Western blotting).

In another embodiment of the invention, the kit of the invention may further comprise a labeled secondary antibody, substrate (when the secondary antibody is an enzyme and labeled), diluents, reaction terminators, instructions and so forth (when the kit is intended to measure the expression of Smad1 and/or a substance having Smad1-activating effect by ELISA).

In still another embodiment of the invention, the kit of the invention may further comprise a color formers, aqueous hydrogen peroxide, buffers, a dyes for counter-staining, instructions and so forth (when the kit is intended to measure the expression of Smad1 and/or a substance having Smad1-activating effect by immunohistochemical analysis).

2. Method and Kit for Detecting Proliferative Diseases Causing Sclerosis

The present invention provides a method of detecting proliferative diseases causing sclerosis, comprising measuring the expression of at least one substance selected from the

group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins in a biological sample.

The term "proliferative diseases causing sclerosis" means diseases where organ sclerosing is observed, and refers to a state where cell proliferation and/or expansion of extracellular matrix is recognized prior to sclerosis or in parallel with sclerosis. Proliferative diseases causing sclerosis include, but are not limited to, renal diseases damaging the glomeruli such as diabetic nephropathy, chronic glomerulonephritis, membranous proliferative glomerulonephritis, focal glomerulosclerosis, light chain disease (L chain deposition disease), lupus nephritis, cryoglobulinemic nephritis, HIV-associated nephritis and purpuric nephritis; hepatic fibrosis; arteriosclerosis; and the like.

Chronic glomerulonephritis is a state of chronic renal disorders, resulting in inflammation and gradual, progressive destruction of the glomeruli. Chronic glomerulonephritis is a syndrome including diseases such as membranous proliferative glomerulonephritis, focal glomerulosclerosis, light chain disease (L chain deposition disease), lupus nephritis, cryoglobulinemic nephritis, HIV-associated nephritis and purpuric nephritis.

Diabetic nephropathy is one of the representative diabetic complications and refers to a state in which renal functions are progressively reduced because of prolonged hyperglycemic conditions caused by diabetes.

Hepatic fibrosis is found in hepatic cirrhosis or chronic hepatitis, and refers to a state in which expansion of the extracellular matrix such as collagen is recognized in places (Disse's spaces) between the hepatic sinusoid wall and the hepatic cords. Hepatic fibrosis is a risk factor for the development of hepatocellular carcinoma, and it is known that progress of fibrosis makes the incidence of hepatocellular carcinoma higher.

Arteriosclerosis, which is a generic term for lesions where the arterial wall becomes thickened or sclerosed, is believed to be chronic inflammatory/proliferative lesions attributable to endothelial cell injuries caused by oxidation stress or the like. When arterial constriction and occlusion occur as a result of progress of arteriosclerosis, rise in blood pressure, myocardial infarction, cerebral infarction, etc. are caused. However, patients have few subjective symptoms prior to organ dysfunction.

The biological sample may be any biological sample as long as at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins is detectable therein. Specific examples of the biological sample which may be used in the invention include renal tissue sections, blood, sera and urine.

STAT3 is one of signal transducer and activator of transcription (STAT) proteins. STAT3 is activated via tyrosine phosphorylation by receptor-associated kinases when various cytokines and growth factors (such as interferon, epithelium growth factor, interleukin 5, interleukin 6, hepatocyte growth factor, leukemia inhibitory factor and bone growth factor 2) have bound to their receptors (phosphorylated STAT).

Phosphorylated Smad1 is a Smad1 which is in an activated state through phosphorylation of its serine residues.

The expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins may be measured at the nucleic acid level (i.e. mRNA expression) and/or the protein level.

With respect to the measurement at the nucleic acid level, total RNA may be extracted from a biological sample, and then the mRNA of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins may be measured by RT-PCR using a pair of appropriate primers. These primers may be designed so that a specific region in sequences such as the nucleotide sequence for human-derived STAT3 mRNA available as NM_139276 in NCBI Refseq database (SEQ ID NO: 19); the nucleotide sequence for human-derived Smad1 mRNA available as NM_005900 in NCBI Refseq database (SEQ ID NO: 1); the nucleotide sequence for the mRNA of human-derived activin receptor-like kinase 1 available as NM_000020 in NCBI Refseq database (SEQ ID NO: 2); the nucleotide sequence for the mRNA of human-derived activin receptor-like kinase 3 available as NM_004329 in NCBI Refseq database (SEQ ID NO: 20); the nucleotide sequence for BMP2 mRNA available as ACCESSION NM_001200 VERSION NM_001200.1 in GenBank database (SEQ ID NO: 3); and the nucleotide sequence for BMP4 mRNA available as ACCESSION NM_001202 VERSION NM_001202.2 in GenBank database (SEQ ID NO: 4) is amplified specifically. Examples of nucleotide sequences for appropriate primer pairs are as described below.

RT-PCR to amplify STAT3 mRNA specifically:

Forward primer: 5'-agatgctcactgcgctgga-3' (SEQ ID NO: 21)

Reverse primer: 5'-tccaatgcaggcaatctgtt-3' (SEQ ID NO: 22)

RT-PCR to amplify Smad1 mRNA specifically:

Forward primer: 5'-ACTACCACCACGGCTTTCAC-3' (SEQ ID NO: 5)

Reverse primer: 5'-AATAGGATTGTGGGGTGAGC-3' (SEQ ID NO: 6)

RT-PCR to amplify ALK1 mRNA specifically:

Forward primer: 5'-ccgtcaagatct tctcctcg-3' (SEQ ID NO: 7)
Reverse primer: 5'-tcatgtctgaggcgatgaag-3' (SEQ ID NO: 8)
RT-PCR to amplify ALK3 mRNA specifically:
Forward primer: 5'-tggcactgggatgaaatca-3' (SEQ ID NO: 23)
Reverse primer: 5'-tggttacataaattggtccga-3' (SEQ ID NO: 24)
RT-PCR to amplify BMP2 mRNA specifically:
Forward primer: 5'- cccagcgtgaaaagagagac-3' (SEQ ID NO: 9)
Reverse primer: 5'- gagaccgcagtccgtctaag-3' (SEQ ID NO: 10)
RT-PCR to amplify BMP4 mRNA specifically:
Forward primer: 5'- tgagcctttccagcaagttt -3' (SEQ ID NO: 11)
Reverse primer: 5'- cttccccgtctcaggtatca -3' (SEQ ID NO: 12)

Alternatively, total RNA may be extracted from a biological sample, and then the mRNA of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins may be measured by Northern hybridization using an appropriate probe. The appropriate probe may be designed based on sequences such as the nucleotide sequence for human-derived STAT3 mRNA available as NM_139276 in NCBI Refseq database (SEQ ID NO: 19); the nucleotide sequence for human-derived Smad1 mRNA available as NM_005900 in NCBI Refseq database (SEQ ID NO: 1); the nucleotide sequence for the mRNA of human-derived activin receptor-like kinase 1 available as NM_000020 in NCBI Refseq database (SEQ ID NO: 2); the nucleotide sequence for the mRNA of human-derived activin receptor-like kinase 3 available as NM_004329 in NCBI Refseq database (SEQ ID NO: 20); the nucleotide sequence for BMP2 mRNA available as ACCESSION NM_001200 VERSION NM_001200.1 in GenBank database (SEQ ID NO: 3); and the nucleotide sequence for BMP4 mRNA available as ACCESSION NM_001202 VERSION NM_001202.2 in GenBank database (SEQ ID NO: 4) so that it specifically hybridizes to a part or the entire region of such sequences. The probe may be labeled with a substance such as ^{32}P.

With respect to the measurement at the protein level, at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins may be measured by a method such as Western blotting, ELISA or immunohistochemical analysis using, for example, antibodies to at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins.

These antibodies may be labeled with a fluorescent dye, enzyme, heavy metal, or the like (direct method). Alternatively, instead of labeling these antibodies, antibodies (secondary antibodies) specific to these antibodies (primary antibodies) may be labeled with a fluorescent dye, enzyme, heavy metal, or the like (indirect method). Preferably, these antibodies are immobilized on solid carriers such as test sections or latex particles.

The expression "measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins" encompasses to detect the presence or absence of the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins and to quantitate the expression level of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins.

According to the present invention, it is possible to detect proliferative diseases causing sclerosis. Briefly, the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins indicates the onset of proliferative diseases causing sclerosis. Conventionally, measurement of urinary type IV collagen and urinary albumin has been used in the diagnosis of renal diseases damaging the glomeruli (such as diabetic nephropathy and chronic glomerulonephritis). The present invention may supersede or supplement such measurement.

Further, according to the present invention, it is possible to evaluate the degree of progress and/or the efficacy of treatment of proliferative diseases causing sclerosis. Briefly, the expression level of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins is proportional to the severity of proliferative diseases causing sclerosis. When the treatment of proliferative diseases causing sclerosis is effective, the expression level of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins decreases keeping pace with the recovery of the patient.

The present invention also provides a kit for detecting proliferative diseases causing

sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins.

Further, the present invention provides a kit for evaluating the degree of progress and/or the efficacy of treatment of proliferative diseases causing sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins.

Proliferative diseases causing sclerosis are as described above.

Examples of reagents for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins include, but are not limited to, a pair of primers capable of amplifying a specific region of the nucleotide sequence of the mRNA of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins; a probe capable of hybridizing to a part or the entire region of the mRNA of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins; and an antibody to at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins. These primer pairs and antibodies are as described above.

The kit of the invention may further comprise reverse transcriptase, DNA polymerase, RNase-free water, buffers, control mRNA, control primer pair, dNTP mix, instructions, and so forth (when the kit is intended to measure the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins at the nuclear acid level using a primer pair).

Alternatively, the kit of the invention may further comprise a transcription buffer, blocking reagent, washing solutions, instructions and so forth (when the kit is intended to measure the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins by Western blotting).

In another embodiment of the invention, the kit of the invention may further comprise a labeled secondary antibody, substrate (when the secondary antibody is an enzyme and labeled), diluents, reaction terminators, instructions and so forth (when the kit is intended to measure the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins by ELISA).

In still another embodiment of the invention, the kit of the invention may further comprise a color formers, aqueous hydrogen peroxide, buffers, a dyes for counter-staining, instructions and so forth (when the kit is intended to measure the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1, phosphorylated Smad1, activin receptor-like kinase 1, activin receptor-like kinase 3 and bone morphogenetic proteins by immunohistochemical analysis).

3. Drugs and Pharmaceutical Compositions

The present invention provides a prophylactic and/or therapeutic agent for proliferative diseases causing sclerosis, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Proliferative diseases causing sclerosis are as described above.

Further, the present invention provides a drug inhibiting the increase of extracellular matrix, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1. Extracellular matrix is a stable biostructure surrounding cells within animal tissues which is an assembly of biopolymers synthesized by cells and secreted/accumulated out of the cells. Extracellular matrix also includes those structures that were synthesized/secreted by cultured cells and deposited around the cells. Extracellular matrix is found abundantly in connective tissues. The basement membrane is also a type of extracellular matrix.

Further, the present invention provides a drug inhibiting the expression of α1 type IV collagen, comprising as an active ingredient a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

These drugs may be used as pharmaceuticals or as reagents for use in experiments.

Example of the substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1

and phosphorylated Smad1 include, but are not limited to, antisense oligonucleotides to Smad1 (one example of such nucleotide sequences is given in SEQ ID NO: 13); SANE (Smad1 Antagonistic Effector) (Raju GP et al., J Biol Chem. 2003 Jan 3;278(1):428-437); anti-PDGF β receptor antibody (APB5); and antisense oligonucleotides to STAT3. Any of the proteins may be produced by the recombinant DNA technology in *Escherichia coli*, yeast, insect cells, animal cells or cell-free protein synthesis systems. Antisense oligonucleotides to Smad1 or STAT3 may be synthesized by known methods in commercial DNA synthesizers. APB5, which is anti-mouse PDGFR-β antibody, may be prepared as follows. Briefly, a cDNA fragment corresponding to the extracellular domain of mouse PDGFR-β was inserted into CD4Rg vector. A fusion protein with human IgG1 (PDGFR-β/Human IgG1) was expressed in COS-1 cell strain. The fusion protein was purified from the culture supernatant and used for immunizing Wistar rats. Fusion cells were prepared using splenic cells from the rats and myeloma cells, followed by selection of cells producing antibodies to PDGFR-β. Not only APB5 but also other anti-PDGFR-β specific antibodies that can be prepared by known methods may be used in the same manner as APB5 is used.

One substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 may be used. Alternatively, a plurality of such substances may be used in combination.

The substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 may be administered alone or together with pharmacologically acceptable carriers, diluents or excipients in appropriate forms of pharmaceutical compositions, to mammals (e.g. human, rabbit, dog, cat, rat, mouse, etc.) orally or parenterally. Dose levels may vary depending upon the patient to be treated, the target disease, symptoms, administration route, and so on. However, in the administration to adult patients, it is convenient to inject a substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 (e.g., SANE) intravenously at a dose of about 10-100 mg/kg body weight, preferably about 60-40 mg/kg body weight per administration about once or twice a month; preferably, the above dose is administered for two or three consecutive days at the beginning of treatment. In other parenteral administration and oral administration, similar dose levels may be used. If symptoms are particularly heavy, the dose may be increased accordingly.

Compositions for oral administration include solid or liquid preparations such as

tablets (including sugar-coated tablets and film-coated tablets), pills, granules, dispersants, capsules (including soft capsules), syrups, emulsions and suspensions. These compositions may be prepared according to conventional methods and may contain carriers, diluents or excipients conventionally used in the field of medicine manufacture. For example, lactose, starch, sucrose, magnesium stearate and the like are used as carriers or excipients for tablets.

Compositions for parenteral administration include, for example, injections and suppositories. Injections include intravenous injections, subcutaneous injections, intradermal injections, muscle injections, instilment injections, etc. Such injections may be prepared by conventional methods, i.e., by dissolving, suspending or emulsifying a substance having an inhibitory effect on the expression of Smad1 in an aseptic, aqueous or oily liquid conventionally used in injections. Examples of aqueous liquids for injection include physiological saline and isotonic solutions containing glucose and other auxiliary agents. They may be used in combination with a suitable auxiliary solubilizer such as alcohol (e.g. ethanol), polyalcohol (e.g. propylene glycol, polyethylene glycol), nonionic surfactant [e.g. Polysorbate 80™, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], etc. Examples of oily liquids for injection include sesame oil and soybean oil. They may be used in combination with an auxiliary solubilizer such as benzyl benzoate, benzyl alcohol, etc. Usually, the prepared injections are filled in appropriate ampoules. Suppositories for administration into rectum may be prepared by mixing a substance having an inhibitory effect on the expression of Smad1 with a conventional suppository base.

It is convenient to formulate the above-described pharmaceutical compositions for oral or parenteral administration into unit dosage forms that would give an appropriate dose of the active ingredient. Examples of such unit dosage forms include tablets, pills, capsules, injections (ampoules), and suppositories.

The above-described pharmaceutical compositions may contain other active ingredients as long as they do not produce undesirable interaction when combined with the substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

When the substance having an inhibitory effect on the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 is an antisense oligonucleotide to Smad1 or STAT3, the antisense oligonucleotide may be introduced into the patient or cells of the patient by known methods of gene transfer. For example, a method in which an antisense oligonucleotide to Smad1 or STAT3 is enclosed in liposomes and then taken into cells ("Lipidic vector systems for gene

transfer" (1997) R.J. Lee and L. Huang Crit. Rev. Ther. Drug Carrier Syst 14, 173-206; Nakanishi M. et al., "Protein, Nucleic Acid and Enzyme" Vol.44, No.11, 1590-1596 (1999)); the calcium phosphate method, electroporation, lipofection, microinjection, a method using a gene gun, and so on may be used. When an antisense oligonucleotide to Smad1 or STAT3 is introduced into cells, a part of the cells at the diseased site may be taken out and then returned to the original tissue after *in vitro* gene transfer. Alternatively, the antisense oligonucleotide may be introduced directly into the tissue of the diseased site.

Pharmaceutical compositions comprising an antisense oligonucleotide to Smad1 or STAT3 as an active ingredient may comprise, if necessary, pharmaceutically acceptable carriers (e.g. diluents such as physiological saline or buffer). Administration of the pharmaceutical composition may be continued until the efficacy of treatment is recognized or until amelioration of conditions is achieved at appropriate dose, with an appropriate administration method and at appropriate frequency, depending on the severity of the target disease and the responsiveness of the patient body.

4. Method and Kit for Identifying Substances Effective in Preventing and/or Treating Proliferative Diseases Causing Sclerosis; Method and Kit for Identifying Substances Effective in Inhibiting the Increase of Extracellular Matrix; and Method and Kit for Identifying Substances Effective in Inhibiting the Expression of α1 Type IV Collagen

The present invention provides a method of identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Proliferative diseases causing sclerosis are as described above.

Further, the present invention provides a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Still further, the present invention provides a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen, comprising judging whether or not a test substance inhibits the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Hereinbelow, one embodiment of the above-described method will be described.

First, cells capable of expressing at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 are

prepared. Any cell capable of expressing at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 may be used. Specific examples which may be used in the invention include mesangial cells derived from renal glomeruli of animals (e.g. those disclosed in Reference S1 described later) and vascular smooth muscle cells.

Cells capable of expressing at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 are cultured in the presence and the absence of a test substance, followed by measurement of the at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1. Examples of the test substance include, but are not limited to, peptides, proteins, non-peptidic compounds, synthetic compounds, fermentation products, cell extracts, plant extracts and animal tissue extracts. These substances may be either novel substances or known substances. The culturing of the cell capable of expressing at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 may be performed under culture conditions suitable for the relevant cell. For example, mesangial cells derived from mouse renal glomeruli (Reference S1 described later) may be cultured as described in Example 1. The method of measuring the expression of STAT3 and Smad1 is as described above.

The expression of phosphorylated STAT3 and phosphorylated Smad1 may be measured by immunostaining using anti-phosphorylated STAT3 antibody (Santa Cruz Biotechnology) and anti-phosphorylated Smad1 antibody (Calbiochem), respectively, as a primary antibody.

The expression level of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 when cells were cultured in the presence of a test substance is compared with the expression level of the at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1 when cells were cultured in the absence of the test substance. When the former is less than the latter, the test substance is judged effective in preventing and/or treating proliferative diseases causing sclerosis; or the test substance is judged effective in inhibiting the increase of extracellular matrix; or the test substance is judged effective in inhibiting the expression of α1 type IV collagen. On the contrary, when the former is equivalent to the latter, or when the former is more than the latter, the test substance is judged ineffective in preventing and/or treating proliferative diseases causing sclerosis; or the test substance is judged ineffective in inhibiting the increase of extracellular matrix; or the test substance is judged ineffective in inhibiting the expression of α1 type IV collagen.

The present invention also provides a kit for identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Further, the present invention provides a kit for identifying substances effective in inhibiting the increase of extracellular matrix, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Still further, the present invention provides a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen, comprising a reagent(s) for measuring the expression of at least one substance selected from the group consisting of STAT3, phosphorylated STAT3, Smad1 and phosphorylated Smad1.

Proliferative diseases causing sclerosis are as described above.

Examples of reagents for measuring the expression of STAT3 or Smad1 include, but are not limited to, primer pairs capable of specifically amplifying a specific region of the nucleotide sequence of STAT3 mRNA or Smad1 mRNA, probes capable of specifically hybridizing to a part or the entire region of STAT3 mRNA or Smad1 mRNA, and antibodies to STAT3 or Smad1. These primer pairs and antibodies are as described above.

Examples of reagents for measuring the expression of phosphorylated STAT3 or phosphorylated Smad1 include, but are not limited to, anti-phosphorylated STAT3 antibody (Santa Cruz Biotechnology) and anti-phosphorylated Smad1 antibody (Calbiochem). These antibodies are as described above.

The kit of the invention may further comprise reverse transcriptase, DNA polymerase, RNase-free water, buffers, control mRNA, control primer pair, dNTP mix, instructions, and so forth (when the kit is intended to measure the expression of STAT3 or Smad1 at the nuclear acid level using a primer pair).

Alternatively, the kit of the invention may further comprise a transcription buffer, blocking reagent, washing solutions, instructions and so forth (when the kit is intended to measure the expression of STAT3 or Smad1 by Western blotting).

In another embodiment of the invention, the kit of the invention may further comprise a labeled secondary antibody, substrate (when the secondary antibody is an enzyme and labeled), diluents, reaction terminators, instructions and so forth (when the kit is intended to measure the expression of STAT3, phosphorylated STAT3, Smad1 or phosphorylated Smad1 by ELISA).

In still another embodiment of the invention, the kit of the invention may further

comprise color formers, aqueous hydrogen peroxide, buffers, dyes for counter-staining, instructions and so forth (when the kit is intended to measure the expression of STAT3, phosphorylated STAT3, Smad1 or phosphorylated Smad1 by immunohistochemical analysis).

Hereinbelow, the present invention will be described specifically with reference to the following Examples. These Examples are provided only for the purpose of illustrating the present invention and are not intended to limit the scope of the invention.

EXAMPLE 1

To identify the protein which binds to the CIV site in the promoter region of the mouse Col4 gene, the present inventors constructed a cDNA library from mouse mesangial cells treated with AGEs. Here, the inventors used a yeast one-hybrid system to isolate a clone that encodes a specific transcription factor from the library, and then identified this clone as encoding Smad1. To confirm the binding of Smad1 to the Col4 promoter *in vivo*, the inventors performed a chromatin immunoprecipitation (ChIP) assay. Precipitated DNA was purified, and the promoter region of the Col4 gene was detected by PCR. Anti-Smad1 antibody precipitated chromatin containing the CIV-1 site from cells stimulated with AGEs (Fig. 1A). In contrast, no precipitation was observed in BSA-exposed cells. The inventors found that Smad4 also binds to the CIV-1 site (Fig. 1A). Next, the inventors examined the transcriptional activity of the Col4 gene by a reporter assay. The inventors constructed a vector by linking the CIV-1 promoter upstream of LacZ, and then cotransfected into COS7 cells with a wild-type Smad1 vector. First, the inventors confirmed the expression of Smad1 by Western blot analysis (Fig. 1B). Phosphorylated Smad1 (pSmad1) was detected in culture supernatant of cells that have been tranfected with the wild-type Smad1 vector. Cotransfection of the wild-type Smad1 resulted in a 18-fold increase in β-galactosidase activity compared with that activity in cells cotransfected with mock vector (Mock) (Fig. 1C). β-galactosidase activity was corrected with luciferase activity, and the β-galactosidase activity in cells cotransfected with the mock vector was taken as the standard. Mock had no effect on the β-galactosidase activity in the cells cotransfected with it. These results suggest that Smad1 is certainly involved in the induction of Col4 gene transcription. Thus, Smad1 transcriptionally regulates the Col4 gene.

To determine whether Smad1 is transcriptionally upregulated by AGEs, the inventors examined the expression of Smad1 in mesangial cells with or without AGEs stimulation. The levels of Smad1 mRNA increased in a time-dependent manner (Fig.2A). Similarly, the levels of Col4 mRNA increased in parallel with the upregulation of Smad1

transcription. In the presence of BSA, however, no change was detected in the expression of Smad1 mRNA or Col4 mRNA. Smad1 is known to be phosphorylated and translocated into the nucleus where it participates in the transcriptional regulation of target genes (11) (12). Therefore, the inventors next examined the issue of whether the phosphorylation and translocation of Smad1 is affected by AGEs treatment in mesangial cells (Fig. 2B). Consistent with the results on mRNA, Smad1 and pSmad1 were distributed throughout mesangial cells with a preferential cytoplasmic localization after a 72-hr incubation in the presence of AGEs. Furthermore, nuclear accumulation of Smad1 and pSmad1 in response to AGEs was observed in the cells 120 hours after AGEs stimulation, while BSA-treatment led to little expression of Smad1 and pSmad1. Similarly, both Smad1 and pSmad1 were detected in extracts from AGEs-treated cells, but not in extracts from BSA-treated cells (Fig. 2C). These findings indicate that the regulation of Col4 is correlated with the expression of Smad1 under AGEs exposure.

To examine the importance of Smad1 in the signaling pathway mediating AGEs-induced overexpression of Col4, the inventors specifically inhibited this pathway with an antisense gene (AS). The AGEs-mediated induction of Smad1 was completely abolished in the presence of the antisense gene, but not in the presence of a control oligo (4-mismatch) (Fig. 3A and 3B). The overexpression of Col4 was remarkably attenuated by the inhibition of Smad1. Smad1 mismatch oligo (control) had no effect on Col4 expression (Fig. 3C). These data indicate that Smad1 plays a critical role in the regulation of Col4 expression. Development and progress of diabetic nephropathy in diabetic patients is a huge clinical problem associated with morbidity and mortality. It is clear that in the current therapy, optimal glycemic control can postpone the development and progress of diabetic nephropathy but can not prevent this disease (1) (2). The antisense oligo to Smad1 remarkably attenuates the AGEs-mediated overproduction of Col4. These findings suggest that blockade of Smad1 signaling may prevent ECM production in mesangial cells in diabetic nephropathy. This effect was observed under prolonged AGEs stimulation. Therefore, Smad1 may be a novel therapeutic target in diabetic complications and be useful in combination with the current therapy. To further elucidate the mechanism of Smad1 expression after AGEs treatment, the inventors investigated the expression of activin receptor-liked kinase1 (ALK1) in mesangial cells. ALK1 is one of the TGF-β receptor family proteins and phosphorylates Smad1 and Smad5 specifically. ALK1 is highly expressed in vascular endothelial cells (13) (14), and may be essential for vascular maturation and stabilization (15) (16). Mutations of ALK1 results in human hereditary hemorrhagic telangiectasia (HHT) type II, also known as Osler-Rendu-Weber syndrome (17).

Recent reports show that ALK1 mediates signals from TGF-β through Smad1 to modulate TGF-β-responsive genes (18) (19). The inventors were able to detect an increase in ALK1 expression in AGEs-treated mesangial cells at both mRNA and protein levels, using an RNase protection assay and Western blot analysis, respectively (data not shown). Finally, the inventors investigated the glomerular expression of Smad1 and ALK1 in human diabetic nephropathy. Indirect fluorescent antibody technique was carried out on renal biopsies (diabetic nephropathy) and on normal kidney tissue. Glomerular immunoreactivities to Smad1 and ALK1 antibodies were proportionate to the severity of sclerotic lesions in glomeruli with diabetic nephropathy: on the other hand, immunoreactive signals were nearly absent in normal glomeruli (Fig. 4). These histological observations suggest that the ALK1/Smad1 signaling pathway is linked to the upregulation of Col4. Since diabetic nephropathy in human is a process that progresses slowly over many years, it is likely that a very detailed evaluation of this phenomenon will be required to elucidate the interaction of Smad1 and ALK1 in this condition.

Targeted gene disruption of Smad1 gene in mice results in embryonic lethality. This suggests that Smad1 plays critical roles in early embryogenesis (20). However, because of the early embryonic lethality, little is known about the role of Smad1 *in vivo*, particularly in the adult. Smad1 is well known to transduce BMP signals (12) and to be especially important in the development of kidney (21). However, Smad1 expression is not detected in glomeruli in adult mice (22). The inventors demonstrated for the first time that AGEs induce the expression of Smad1 in adult mouse glomeruli. The inventors observed that chronic exposure to AGEs, inducing sustained increase in Smad1 expression, leads to Col4 overproduction and suggested that Smad1 is a critical modulator in diabetic conditions. Since AGEs are significantly involved in diabetic complications, the results obtained by the inventors may give valuable insights into any disease and condition where collagen deposition occurs, such as diabetes or aging. Changes in GBM structure occur very early in diabetic nephropathy, even before microalbuminuria is apparent. Therefore, in diabetic nephropathy, Smad1 may be the earliest indicator of renal dysfunction. Recent reports demonstrated that ALK1 mediates signals from TGF-β via Smad1 (18, 19). Therefore, the inventors investigated the expression of ALK1 in mouse mesangial cells and human kidney tissues. As a result, the inventors demonstrated that ALK1 and Smad1 are expressed in renal glomeruli in response to the progress of diabetic conditions. These results lead to the development of novel therapeutic strategies for the treatment of diabetic complications in various organs by suppressing the pathologically activated production of collagen (1). This confirms that sustained hyperglycemia, reflected by an increase in AGEs, is a prerequisite for

the development of long-term diabetic complications (23, 24). Glycation leads ultimately to increased crosslinking of collagen resulting in increased arterial stiffness (25). Moreover, the correlation between AGEs and the development of diabetic complications and arteriosclerosis has been recently emphasized by studies using specific AGEs inhibitors (26, 27). Although Col4 is the principal component of the vascular basement membrane, the cellular and molecular mechanisms involved in the upregulation of Col4 in diabetic conditions or aging are as yet poorly understood. The inventors here elucidate that Smad1 directly regulates Col4 gene expression. Accordingly, the inventors speculate that the ALK1/Smad1 signaling pathway may mediate the development of arteriosclerosis, both in diabetic patients and in the aged, by inducing an overproduction of ECM. Further work is in progress to clarify the role of the ALK1/Smad1 signaling pathway in diabetic or aged animal models.

Further, mRNA expression levels in mesangial cells cultured in the presence of AGEs were compared with corresponding mRNA expression levels in mesangial cells cultured in the presence of BSA (Fig. 5). In the presence of AGEs, transcription of BMPRII and BMP4 was remarkably enhanced. Although no big change was recognized in Smad1 transcription level, big changes in its transcription level are difficult to perceive because Smad1 is a transcription factor. Besides, it is believed that translocation from the cytoplasm to the nucleus and phosphorylation (which are important for the effect of a transcription factor) are not reflected in the experimental results using microarrays.

Urinary BMP2 levels in a diabetic nephropathy patient were determined by Western blotting. The results revealed that urinary BMP2 was reduced as the disease was improved by treatment (Fig. 6).

Chronic stimulation with TGF-β signals promoted expression of BMP2 and BMP4 proteins remarkably (Fig. 7). This suggests that these BMP proteins perform central functions in the TGF-β signaling pathway.

Materials and Methods

Cell culture

A glomerular mesangial cell strain was established from glomeruli isolated from normal, 4 week-old mice (C57BL/6JxSJL/J), and was identified according to the method previously described (S1). The mesangial cells were cultured in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) supplemented with 1 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 20% fetal calf serum. The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells (S2).

AGEs or BSA exposure was carried out as described previously (S3).

cDNA library construction and Yeast One-Hybrid screening

The inventors prepared cDNA from mouse mesangial cells exposed to AGEs and inserted it into pGAD vector. Yeast one-hybrid screening was carried out using MATCHMAKER one-hybrid kit (Clontech, Palo Alto, California). Briefly, tandem repeats of the 27 bp sequence (TTCCTCCCCTTGGAGGAGCGCCGCCCG: CIV-1) (SEQ ID NO: 14) from the mouse Col4 gene were ligated into a yeast integration and reporter vector pHISi (MATCHMAKER One-hybrid: Clontech, Palo Alto, California) or pLacZi (MATCHMAKER One-hybrid: Clontech, Palo Alto, California) to generate CIV-1-pHISi or CIV-1-pLacZi vector, respectively (S4). Each of these reporter constructs was linearized and integrated into the chromosome of yeast YM4271 (MATCHMAKER One-hybrid: Clontech, Palo Alto, California). The resulting yeast cells with the integrated CIV-1-pHISi and CIV-1-pLacZi were used for one-hybrid screening with the AGEs stimulated-mouse mesangial cell-derived cDNA library. Positive colonies were selected on SD/-His/-Leu plates with 45 mM 3-amino-1, 2, 4-triazole (3-AT). To exclude false positive clones, the inventors performed β-galactosidase filter lift assay (Clontech). Plasmids were rescued from the remaining yeast colonies and retransformed into *E. coli* DH5α.

ChIP assay

ChIP assays were essentially performed as described previously by Luo et al (S5). The inventors used anti-Smad1 antibody, anti-Smad4 antibody (Santa Cruz Biotechnology, Santa Cruz, California) or normal control IgG at 4 °C overnight. PCR was performed to amplify the region containing the CIV-1 motif. The 5' primer was 5'-GGAGCTCCCCAATTTGTTG-3' (SEQ ID NO: 15), and the 3' primer was 5' –CAGCCTCCGCCTCTTACC-3' (SEQ ID NO: 16). The resulting PCR product was around 100 bp on agarose gel electrophoresis.

Reporter assay

1.3 X 10^5 COS7 cells in 10% fetal bovine serum-added Dulbecco's modified Eagle's Medium (DMEM) were seeded into six-well plates. Eight hours later, the cells were cotransfected with 750 ng of CIV-1-LacZ reporter construct along with either 750 ng of vector encoding wild type Smad1 or a mock vector. 75 ng of CMV-LUC (Firefly luciferase gene under the control of CMV promoter) was also introduced into the cells as an internal control. Transfection was performed with FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, Indiana). Forty-eight hours later, the cells ware harvested in reporter lysis buffer. Then, β-galactosidase and luciferase activities were measured using β-galactosidase Reporter System (BD Biosciences, San Jose, California) and

Luciferase Reporter Assay System (Promega, Madison, Wisconsin). β-galactosidase results were corrected with luciferase activities measured.

RNase protection assay

RNase protection assay was performed as described previously (S6). The nucleotide sequence of the probe used in this assay corresponds to positions 1172-1433 of Acc No. U58992, as described below:

cccaccacc gtctgcaaga tccccagcgg gtgcagcttg aaaatcttca acaaccaaga gtttgctcag
ctactggcgc agtctgtgaa
ccacgggttc gagaccgtgt atgaactcac caaaatgtgc actattcgga tgagcttcgt
gaagggttgg ggagccgaat accaccggca ggatgttacc agcacccccct gctggattga
gatccatctg catggccctc tccagtggct ggataaggtt ctgacccaga tgg
(SEQ ID NO: 17)

Western blotting

Mesangial cells were cultured in the presence of AGEs or BSA (as control) for 72 hours. Cells were harvested in sample buffer, resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitro-cellulose membrane and subjected to Western blot using a 1:500 dilution of anti-Smad1 antibody and anti-pSmad1 antibody (Santa Cruz Biotechnology), followed by detection using an enhanced chemiluminescence detection system (Invitrogen, Carlsbad, California).

Immunostaining of cultured cells and cytosections

Cultured cells were fixed in 4% paraformaldehyde. The following antibodies were used: anti-Smad1 antibody, 1:100 (Santa Cruz Biotechnology); anti-pSmad1 antibody, 1:100 (Calbiochem). An appropriate fluoresceine isothiocyanate-conjugated secondary antibody was used for visualization and imaging was done using a laser microscope and a fluorescent microscope (Olympus, Tokyo, Japan).

Smad1 morpholino antisense oligonucleotide

The antisense oligonucleotide used was a 25-nucleotide morpholino oligo (Genetools LLC, Philomath, Oregon). The sequence is 5'-CAAGCTGGTCACATTCATAGCGGCT-3' (SEQ ID NO: 13). As a control, an oligo with the base composition 5'-CAtGCTcGTCACATTCAaAGCcGCT-3' (SEQ ID NO: 18) was used. *In vitro* RNA transcription was performed as previously described (S7).

Histology

Histopathological studies were performed on human tissues. This experiment was in accordance with the Declaration of Helsinki, and the inventors obtained approval from the institutional review board. All patients gave their informed written consent. Diabetic

nephropathy renal specimens (n=5) were obtained from renal biopsies. Control human tissue sections were obtained from normal renal cortex harvested from kidneys removed for renal malignancy. Tissues for analysis were sampled from the pole opposite the tumor. Cryopreserved kidney tissues were cut into 5 μm thick sections and fixed in acetone for 5 min. Endogenous peroxidase activity was quenched by a 20 min-incubation in the dark with 1% H_2O_2 in methanol. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at room temperature, followed by immunostaining with primary antibodies: anti-Smad1 (Santa Cruz Biotechnology) and anti-ALK1 (R&D, McKinley Place, Nebraska) antibodies.

Analysis of expression levels with microarrays

Individual mRNA expression levels in mesangial cells cultured in the presence of AGEs and mesangial cells cultured in the presence of BSA were measured using Agilent Technologies Mouse cDNA Microarray Kit.

EXAMPLE 2

Glomerulosclerosis is characterized by quantitative increase in extracellular matrix (ECM). Type IV collagen (Col4) is one of the major components of expanded ECM in glomerular diseases. However, the molecular mechanism of transcriptional regulation of Col4 gene was not clear until the recent report of the present inventors. The inventors showed that Smad1 transcriptionally regulates the overexpression of Col4 in diabetic nephropathy (A8). Smad1 directly transduces signals to downstream target genes, such as osteopontin (A9), inhibition of differentiation (A10), and type I collagen (A11), and is essentially important for the development and progress of kidney diseases (A12). These findings suggest that Smad1 is a transcriptional factor critical for the development and progress of glomerulosclerosis.

Signal transducer and activation (STAT) proteins were shown to be involved in signal transduction of numerous cytokines and growth factors. STAT3 activation is a key regulator for PDGF-induced mitogenesis (A13). Nakashima et al reported that transcriptional coactivator p300 physically interacts with STAT3 and Smad1, which were followed by the subsequent activation of the target gene transcription in astrocyte differentiation (A14). The inventors postulated from these findings that PDGF activates the STAT3-Smad1 signaling pathway in mesangial cell proliferation and that this process is essential for mesangial cells to progress into glomerulosclerosis.

In this study, the inventors demonstrated the effect of administration of anti-PDGF β-receptor antibody that inhibits activation by PDGF-B chain in rat glomerulonephritis, and

examined the signaling pathway for regulating both glomerular cell proliferation and glomerulosclerosis *in vivo* and *in vitro*.

Materials and Methods

Animals

Male Wistar rats (CLEA Japan, Inc. Japan) weighing 180 to 200 g were used for this study. Rats were raised under specific pathogen-free conditions. All of the animal experiments were performed in accordance with institutional guidelines, and the Review Board of Tokushima University granted ethical permission to this study.

Induction of Thy1 glomerulonephritis

Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody (1mg/kg) (Cedarlane Laboratories, Ontario, Canada) as described elsewhere (A15). These rats were sacrificed at days 1, 2, 4, 6, and 12 (n=6 per group) after the administration of anti-Thy-1.1 antibody. Six age-matched rats were injected with vehicle alone and sacrificed as controls.

Protocol of treatment with anti-PDGF β-R antibody in Thy1 GN

A rat monoclonal anti- PDGF β-receptor antibody (APB5) and its antagonistic effects on the PDGF β-R signal transduction pathway *in vivo* and *in vitro* were described previously (A16, A17). The rats were injected intraperitoneally everyday with 400 μg of APB5 (kindly provided by Prof. Shinichi Nishikawa of RIKEN) or irrelevant isotype-matched control rat IgG (kindly provided by Prof. Shinichi Nishikawa of RIKEN) after the administration of anti-Thy1.1 antibody from day 0, and were sacrificed at days 1, 2, 4, 6, and 12 (n=6 per group).

Histological examination

Light microscopy

After removal of the kidney, tissue blocks for light microscopy study were fixed in methyl Carnoy's solution (methanol: glacial acetic acid=3:1), and embedded in paraffin. Sections (2 μm) were stained with hematoxylin and eosin (HE), periodic acid-Schiff's reagent (PAS) and periodic acid-methenamine silver (PAM).

Immunohistochemistry

Kidney sections were processed for immunohistochemistry according to standard procedures. For studying proliferating cell nuclear antigen (PCNA), Col4 and Smad1, methyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 minutes to deactivate endogenous peroxidase. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 minutes at room temperature, and

then incubated with Avidin D blocking solution and Biotin blocking solution (Vector, Burlingham, CA, USA) for 15 minutes each. Sections were incubated with anti-PCNA antibody (1: 200 dilution), anti-Col4 antibody (1: 200 dilution), and anti-Smad1 antibody (1: 100 dilution) (Santa Cruz Biotechnology, CA, USA) for 60 minutes at room temperature, and then incubated with appropriate biotinylated secondary antibodies followed by incubation with avidin- biotin peroxidase complex (Vectastain ABC System, Vector). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride (DAB). For studying phosphorylated Smad1 (pSmad1) and phosphorylated (pSTAT3), tissues were snap-frozen in cold acetate in OCT compound (Miles Inc., IN, USA), and were cut into 4 μm-thick sections and fixed in acetone for 5 minutes, and treated with 0.3% hydrogen peroxide in methanol for 30 minutes to deactivate endogenous peroxidase. Sections were treated in the same manner as sections for PCNA examination were treated, with the following primary antibodies: anti-pSmad1 antibody (1: 100 dilution) (Calbiochem, CA, USA) and anti-pSTAT3 antibody (1: 100 dilution) (Santa Cruz Biotechnology). To evaluate the nuclear number, sections were counterstained with hematoxylin solution.

Quantitation of light microscopy

Glomerular morphometry was performed on PAM-stained tissues. The glomerular surface area and the PAM-positive area to glomerular area (%) were measured using an image analyzer with a microscope (IPAP; Image Processor for Analytical Pathology; Sumitomo Chemical Co., Osaka, Japan) as described (A18-A20). For each animal, 50 glomeruli were analyzed.

Quantitation of immunohistochemistry

PCNA: For quantitation of proliferating cells (PCNA positive cells), a blinded observer evaluated 50 glomeruli per specimen and mean values per glomerulus were calculated. pSmad1: To quantitate the expression of pSmad1, pSmad1 positive cells per glomerular cell were counted, and mean percentages of pSmad1 positive cells were calculated. Col4, Smad1 and pSTAT3: The area stained brown on an immunoperoxidase-stained section was selected for its color range, and the percentage of this area to total glomerular mesangial area was quantitated by using IPAP. In each animal, 50 glomeruli were evaluated.

Cell culture experiment

A glomerular mesangial cell strain was established from glomeruli isolated from normal, 4 week-old mice (C57BL/6JxSJL/J) according to the method previously described (A21). The mesangial cells were cultured in B medium (a 3:1 mixture of minimal essential

medium/F12 modified with trace elements) supplemented with 1 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 20% fetal calf serum (FCS). The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells (A22). The cultured mesangial cells in B medium/20% FCS were plated onto 100 mm dishes. After 24 hours of incubation, the cells were starved for two days in B medium/0.1%BSA, and cultured in B medium/2% FCS with 5ng/ml PDGF-B (Calbiochem), then incubated with100 ng/ml of APB5 or rat IgG (control) for 24 hours.

Cell proliferation test by BrdU ELISA

The proliferation of mesangial cells was also determined using a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis (Amersham Pharmacia Biotech Inc., NJ, USA). The BrdU ELISA was performed according to the manufacturer's instructions. Briefly, mesangial cells were plated at low density in 96-well flat-bottomed microtiter plates containing B medium/10% FCS and allowed to adhere overnight. The subconfluent cells were then starved for two days in B medium/0.1% BSA. 100ng/ml of APB5 was then added to cells in B medium/2% FCS with 5ng/ml of PDGF-B and 10mM BrdU. After six hours of culture, plates were centrifuged and cells denatured with fixative solution then incubated for 30 min with 1:100 diluted anti-BrdU mAbs labeled with peroxidase. After removing the labeled antibody, substrate solution was added for 15 min and the reaction stopped by adding 1 M sulfuric acid. The absorbance was measured within 5 min at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader (Model 550, Bio-Rad Laboratories, CA, USA). The blank corresponded to 100 μl of culture medium without BrdU.

Western blot analysis

Cultured mesangial cells were starved for 24 hours in B medium/0.1% BSA. The cells were stimulated by 5 ng/ml of PDGF-BB with 100 ng/ml of APB5 or control IgG for 120 min. Cells were suspended in lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitro-cellulose membrane and subjected to Western blot using a 1:1000 dilution of anti-pSTAT3 antibody, 1:1000 dilution of anti-pSmad1 antibody and 1:2000 dilution of anti-Col4 antibody, followed by detection using an enhanced chemiluminescence detection system (Amersham Pharmacia).

Cell transfection

Plasmid construct expression vectors of wild type STAT3 and dominant negative STAT3 were kindly provided by Jackie Bromberg (The Rockefeller Univ.) (A23). Mesangial cells (60mm dish) were transfected with an expression vector encoding wild type STAT3 (8mg) or dominant negative STAT3 (8mg) by using Lipofectamine2000 (Invitrogen

Life Technologies) according to the manufacturer's instructions. After 6 hr of transfection, medium was changed to growth medium (60% DMEM, 20% F12, 20% fetal calf serum). After 48 hr, cells were suspended in lysis buffer, and Western blot analysis was performed as previously described.

Statistical analysis

All values were expressed as the mean±SE and analyzed by Mann-Whitney nonparametric analysis, or one-way analysis of variance with a modified t-test. P values < 0.05 were considered significant.

Statistical analyses of cell proliferation test and expression of Smad1 mRNA in cultured mesangial cells were performed by t-test. Quantitation of immunohistochemistry and expression of Smad1 mRNA in glomeruli were analyzed by one-way ANOVA followed by the post hoc test. P values <0.05 were considered significant. Data are expressed as means±SD.

Experimental Results

Morphological changes in Thy1 GN

In Thy1 GN, proliferation of mesangial cell begins at day 2, peaked at day 6, and subsides at day 12 after the injection. Fig. 9 shows a representative light microscopic picture at day 6 in each group. Thy1 GN group showed increase of the mesangium, which was peaked at day 6 (Fig. 9B). Proliferation of glomerular cells was assessed by immunostaining of PCNA. PCNA positive cells were markedly increased in Thy1 GN group, and peaked at day 6 (Fig. 9E).

Col4 is one of the main components of ECM in glomerulosclerosis. Col4 was weakly visible along the glomerular basement membrane and almost negative in the glomeruli in the normal control group (Fig. 9G). On the other hand, Thy1 GN group showed strong Col4 positive in the expanded mesangial area (Fig. 9H).

In Thy1 GN, both PDGF-B and PDGF β-receptor were significantly positive in the glomeruli (Fig. 10). These findings indicate that excessive proliferation of mesangial cells, glomerular hypertrophy and glomerulosclerosis lesions occur coincidently in glomerulonephritis induced by anti-Thy1 antibody.

Anti-PDGF β-receptor antibody inhibits both glomerular cell proliferation and glomerulosclerosis *in vivo*

APB5 inhibits PDGF β-R-mediated signaling pathways as described previously. Treatment with APB5 showed significant reduction in both glomerular cell number and

glomerular PCNA positive cells in Thy1 GN at each point examined (Fig. 9C, 9F, 11A, 11B). Overexpression of PDGF-B chain and PDGF β-R were significantly reduced after administration of APB5 (Fig. 10C, 10F). APB5 treatment also reduced mesangial matrix increase in Thy1 GN, which was assessed with the ratio of PAM-positive area to glomerular area (Fig. 11C). Col4 expression in mesangial cells in Thy1 GN was reduced by APB5 treatment (Fig. 11D). These data indicate that APB5 treatment can reduce both the mesangial cell proliferation and the mesangial matrix expansion in Thy1 GN.

Time course of expression of Smad1, phosphor-Smad1 (pSmad1) and phophor-STAT3 (pSTAT3) in Thy1 GN

The inventors examined the expression of Smad1 in the Thy1 GN rat kidney by immunostaining. Although Smad1 was hardly detected in healthy control glomeruli (Fig. 12A), a typically expanded mesangial pattern was observed in the glomeruli of Thy1 GN group at day 6 with high expression of Smad1 there (Fig. 12B). IPAP image analysis system was used to quantitate the expression of Smad1. The peak of glomerular Smad1 expression occurred at day 6 (Fig. 13A), which was consistent with the peak of mesangial cell proliferation. As shown in Fig. 12C, glomerular Smad1 expression declined rapidly after day 6.

Subsequently, the inventors examined whether or not the transcription and phosphorylation of Smad1 are occurring in Thy1 GN. As a result of immunohistochemistry, pSmad1 was hardly observed in healthy control group (Fig. 14A). However, in Thy1 GN group, pSmad1 was strongly positive in the nuclei (Fig. 14B). To quantitate the expression of pSmad1, pSmad1 positive cells per glomerulus were counted (Fig. 13B). Glomerular expression of pSmad1 was upregulated at day 1 of Thy1 GN and reached the peak at day 4, which was the early phase of mesangial cell proliferation.

Since PDGF-B and PDGF β-R were upregulated in Thy1 GN and APB5 inhibited the overexpressions thereof, the inventors performed immunostaining of phosphorylated STAT3 which is a transcription factor of PDGF signaling pathway (A24). The expression of pSTAT3 was extensively increased in Thy1 GN (Fig. 15A, 15B, 15C), and peaked at day 6 (Fig. 13C).

APB5-treated groups had a significantly reduced expression of Smad1 and pSmad1 proteins in the glomeruli in Thy1 GN (Fig. 12D, 12E, 14D, 14E, 16A, 16B). Overexpression of pSTAT3 was also significantly reduced after administration of ABP5 at every point examined (Fig. 15D, 15E, 16C).

Effect of anti-PDGF β-R antibody *in vitro*

To determine whether or not APB5 inhibits the proliferation of mesangial cells, the

inventors examined the proliferation of mesangial cells with or without APB5 by using BrdU ELISA system. As shown in Fig. 17A, addition of APB5 suppressed the PDGF-induced DNA synthesis in mesangial cells.

The inventors studied whether APB5 inhibits the expression of pSTAT3, pSmad1 and Col4 in mesangial cells stimulated by PDGF-B using Western blot analysis. APB5 reduced phosphorylation of STAT3 and Smad1 and the expression of Col4 (Fig. 17B).

Interaction between STAT3 and Smad1

To elucidate the interaction between STAT3 and Smad1 that increases Col4 expression, a vector encoding dominant negative STAT3 was introduced into cultured mesangial cells.

The introduction of dominant negative STAT3 definitely reduced the expression of pSmad1 and Col4 compared with the introduction of wild type STAT3 (Fig. 18).

Discussion

Many glomerular disorders are characterized by both mesangial cell proliferation and progressive glomerulosclerosis. However, mechanisms common for both of these important pathological findings have not been elucidated to date. This study demonstrated for the first time that activation of STAT3 and Smad1 is in a key pathway for regulating the interaction between the two critical events of progressive glomerular disorders. These results support a new direction of research about the pathogenesis and its therapeutical approach for chronic glomerulonephritis and diabetic nephropathy which are major problems in the 21st century in the world.

Glomerulosclerosis is a pathological feature seen in progressive glomerular disorders including chronic glomerulonephritis, IgA nephropathy and diabetic nephropathy. Glomerular cell proliferation occurs at an early stage in a number of glomerular diseases and subsequently glomerulosclerosis develops, which eventually progresses end stage glomerular disorders (A1, A2). Examples of this process are seen in IgA nephropathy, membranoproliferative glomerulonephritis, diabetic nephropathy, and light chain systemic diseases in human as well as in animal models such as Thy1 GN rat renal ablation model and so on (A25, A26). Inhibiting glomerular cell proliferation with anti-PDGF antibody (A7), anti-coagulant heparin (A27) or vitamin D analogue (A19) demonstrated to abolish the subsequent development of progressive glomerulosclerosis, but the mechanism has been unclear. In this study, the inventors have demonstrated the possible mechanism regulating the interaction between mesangial cell proliferation and glomerulosclerosis for these pathological processes.

A receptor for PDGF has been identified in murine and human mesangial cells

(A28). PDGF is a potent, key mitogen for mesangial cells, and is constitutively synthesized as an autocrine cell growth factor in these cells *in vitro* (A28, A29). PDGF plays an important role for the progress of pathological conditions including glomerulonephritis, diabetic nephropathy and progressive glomerulosclerosis *in vitro* and *in vivo* (A3, A4). It has been previously reported that activation of PDGF receptor tyrosine kinase induces tyrosine phosphorylation of STAT3 proteins (A30, A31). The activation is associated with growth regulation and differentiation (A32, A33). The inventors have demonstrated that the overexpression of phosphorylated STAT3 has been identified associated with increased expressions of both PDGF and its β-receptor *in vivo* and *in vitro*, and that APB5 has ameliorated glomerulonephritis by reducing the expression of PDGF, its β-receptor and STAT3 *in vivo* and *in vitro*.

Glomerulosclerosis is characterized mainly by increase in the amount of ECM in the mesangium. One of the major components of glomerulosclerosis is Col4 (A34). The inventors have recently reported that Smad1 is a key transcriptional factor for regulating Col4 expression in diabetic nephropathy *in vitro* and *in vivo* (A8). The inventors have demonstrated that phosphorylated Smad1 is strongly expressed in parallel with the upregulation of Col4 expression and the increase in the amount of glomerular ECM. These findings elucidate that Smad1 plays a critical role not only in glomerulosclerosis in diabetic nephropathy but also in glomerulonephritis. This study has also shown that PDGF induces expression of phosphorylated Smad1 in the glomeruli *in vitro* and *in vivo*.

The inventors confirmed that the interaction between STAT3 and Smad1 regulates a gene critical for glomerulosclerosis. Introduction of dominant negative STAT3 decreased the expression of Col4 significantly in cultured mesangial cells. Activation of STAT3 and activation of Smad1 seem to be independent but both factors were activated by PDGF. Furthermore, since introduction of dominant negative STAT3 partially reduced phosphorylation of Smad1, activation of Smad1 seems to be a part of the mechanism of activating SMAT3. These findings suggest that, in experimental glomerulonephritis, PDGF-induced STAT3 activation interacts with overexpression of Smad1, which is followed by activation of Col4. To understand both signaling pathways is essential for elucidating the pathological process of progressive glomerular disorders.

Therapeutical approach for sclerosis in diverse organs is currently limited to supportive therapy to slow the loss of function of these organs. The findings of the present inventors offer insights into the nature of even other proliferative diseases that lead to sclerosis. Since both Smad1 and STAT3 are nearly absent in normal glomeruli, blocking Smad1 and/or STAT3 signals may be beneficial to inhibit the progress of various renal

diseases leading to sclerosis, by inhibiting the pathologically activated cell proliferation and production of ECM.

EXAMPLE 3

Urine samples from five patients with diabetic nephropathy, one patient with diabetes complicated with sclerosing nephritis, two patients with diabetes complicated with non-sclerosing nephritis, and two healthy persons were subjected to SDS-polyacrylamide gel electrophoresis, followed by blotting on nitrocellulose membrane. Western blotting was performed using anti-Smad1 antibody (Santa Cruz Biotechnology) and anti-ALK1 antibody as primary antibodies and Western Breeze kit (Invitrogen, Tokyo, Japan).

Urine samples taken from one inpatient with diabetic nephropathy prior to treatment and one week after start of the treatment were subjected to Western blotting in the same manner, using anti-ALK1 antibody as a primary antibody.

While Smad1 and ALK1 were detected in urine samples from patients with diabetic nephropathy and patient with glomerulosclerosis in the kidney, they were not detected in urine samples from normal persons and nephritis patients without glomerulosclerosis (Figs. 17 and 19). The amount of ALK1 excreted into urine decreased in a time-dependent manner as treatment of diabetic nephropathy progressed (Fig. 18).

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All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

INDUSTRIAL APPLICABILITY

According to the present invention, Smad1 has been identified as a substance directly involved in the overproduction of type IV collagen and shown to have a critical role as a causative of diabetic nephropathy. With this finding, it has become possible to detect diabetic nephropathy; and there have been provided a prophylactic and/or therapeutic agent for diabetic nephropathy, a drug inhibiting the increase of extracellular matrix, and a drug inhibiting the expression of α1 type IV collagen. Further, according to the present invention, there have been provided a method and a kit for identifying substances effective in preventing and/or treating diabetic nephropathy, a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix, and a method and a kit

for identifying substances effective in inhibiting the expression of α1 type IV collagen.

According to the present invention, it has been demonstrated that activation of STAT3 and Smad1 is in a key pathway for regulating the interaction between the two critical events (i.e., cell proliferation and glomerulosclerosis) in progressive glomerular disorders. With this finding, it has become possible to detect proliferative diseases causing sclerosis; and there have been provided a prophylactic and/or therapeutic agent for proliferative diseases causing sclerosis, a drug inhibiting the increase of extracellular matrix, and a drug inhibiting the expression of α1 type IV collagen. Further, according to the present invention, there have been provided a method and a kit for identifying substances effective in preventing and/or treating proliferative diseases causing sclerosis, a method and a kit for identifying substances effective in inhibiting the increase of extracellular matrix, and a method and a kit for identifying substances effective in inhibiting the expression of α1 type IV collagen.

SEQUENCE LISTING FREE TEXT

<SEQ ID NO: 1>

SEQ ID NO: 1 shows the nucleotide sequence of the mRNA of human-derived Smad1.

<SEQ ID NO: 2>

SEQ ID NO: 2 shows the nucleotide sequence of the mRNA of human-derived ALK1.

<SEQ ID NO: 3>

SEQ ID NO: 3 shows the nucleotide sequence of the mRNA of human-derived BMP2.

<SEQ ID NO: 4>

SEQ ID NO: 4 shows the nucleotide sequence of the mRNA of human-derived BMP4.

<SEQ ID NO: 5>

SEQ ID NO: 5 shows the nucleotide sequence of the forward primer used in RT-PCR for specifically amplifying the mRNA of Smad1.

<SEQ ID NO: 6>

SEQ ID NO: 6 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of Smad1.

<SEQ ID NO: 7>

SEQ ID NO: 7 shows the nucleotide sequence of the forward primer used in

RT-PCR for specifically amplifying the mRNA of ALK1.

<SEQ ID NO: 8>

SEQ ID NO: 8 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of ALK1.

<SEQ ID NO: 9>

SEQ ID NO: 9 shows the nucleotide sequence of the forward primer used in RT-PCR for specifically amplifying the mRNA of BMP2.

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SEQ ID NO: 10 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of BMP2.

<SEQ ID NO: 11>

SEQ ID NO: 11 shows the nucleotide sequence of the forward primer used in RT-PCR for specifically amplifying the mRNA of BMP4.

<SEQ ID NO: 12>

SEQ ID NO: 12 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of BMP4.

<SEQ ID NO: 13>

SEQ ID NO: 13 shows the nucleotide sequence of an antisense oligonucleotide to Smad1.

<SEQ ID NO: 14>

SEQ ID NO: 14 shows the nucleotide sequence of the 27 bp tandem repeat sequence of mouse-derived Col4 gene.

<SEQ ID NO: 15>

SEQ ID NO: 15 shows the nucleotide sequence of the 5' primer used in ChIP assay.

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SEQ ID NO: 16 shows the nucleotide sequence of the 3' primer used in ChIP assay.

<SEQ ID NO: 17>

SEQ ID NO: 17 shows the nucleotide sequence of the probe used in RNase protection assay.

<SEQ ID NO: 18>

SEQ ID NO: 18 shows the nucleotide sequence of a synthetic oligonucleotide.

<SEQ ID NO: 19>

SEQ ID NO: 19 shows the nucleotide sequence of the mRNA of human-derived STAT3.

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SEQ ID NO: 20 shows the nucleotide sequence of the mRNA of human-derived ALK3.

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SEQ ID NO: 21 shows the nucleotide sequence of the forward primer used in RT-PCR for specifically amplifying the mRNA of human-derived STAT3.

<SEQ ID NO: 22>

SEQ ID NO: 22 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of human-derived STAT3.

<SEQ ID NO: 23>

SEQ ID NO: 23 shows the nucleotide sequence of the forward primer used in RT-PCR for specifically amplifying the mRNA of human-derived ALK3.

<SEQ ID NO: 24>

SEQ ID NO: 24 shows the nucleotide sequence of the reverse primer used in RT-PCR for specifically amplifying the mRNA of human-derived ALK3.